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1. Chien, J. et al. Mol. and Cell. Endocrinology (2001) 181(1-2): 69-79
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Regulatory peptides and other neuroendocrine markers in medullary carcinoma of the thyroid

F W F Hanna, J E S Ardill, C F Johnston, R T Cunningham, W J Curry, C F J Russell[1] and K D Buchanan

Wellcome Research Laboratories, Department of Medicine, Queen's University of Belfast, Belfast BT12 6BJ, UK and [1]Department of Endocrine Surgery, Royal Victoria Hospital, Belfast BT12 6BJ, UK

(Requests for offprints should be addressed to K D Buchanan)
(F W F Hanna is now at Department of Medicine, Level B7, University Hospital of Wales, Heath Park, Cardiff CF4 4XN, UK)

Abstract

Medullary thyroid carcinoma (MTC) is an APUDoma (APUD refers to amine precursor uptake and decarboxylation) arising from the parafollicular cells. Diarrhoea has been reported in some 30% of patients, variously attributed to excess production of calcitonin (CT), serotonin (5-HT), vasoactive intestinal peptide (VIP) or other factors.

The regulatory factors in MTC were examined employing immunocytochemistry and RIA to tumours and their extracts. The patients were followed up for more than 15 years. CT and calcitonin gene-related peptide were universally expressed in all the tumours. The neuroendocrine markers chromogranin A (and its fragments pancreastatin and WE-14), neurone-specific enolase, protein gene product 9·5 and carcino-embryonic antigen were found in the majority of MTCs and might be useful as immunocytochemical markers. 5-HT, substance P, neurokinin A, glucagon and VIP could not be detected, excluding them as candidates in the diarrhoea of MTC.

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Introduction

Since its identification as a distinct form of thyroid cancer (Hazard *et al.* 1959) and the demonstration that it arises from the parafollicular cells (Williams 1966), subsequently shown to belong to the amine precursor uptake and decarboxylation (APUD) system (Pearse 1968), medullary thyroid carcinoma (MTC) continues to attract interest with respect to its endocrinology.

MTC represents 5–10% of all thyroid malignancies. Eighty per cent of cases are sporadic; in the remaining 20% of patients the tumour is inherited on an autosomal dominant basis in one of three possible forms: (a) multiple endocrine neoplasia (MEN) 2A (MTC, phaeochromocytoma and hyperparathyroidism), (b) MEN 2B (MTC, phaeochromocytoma, mucosal neuromas and gastrointestinal ganglioneuromatosis) and (c) non-MEN familial MTC (de Bustros & Baylin 1991).

MTC has peculiar biochemical and genetic features which are still largely unexplored and its distinct clinical features are, to date, far from explained. As APUDomas, various neuropeptides have been reported to originate from MTCs but their relationship to symptomatology are not documented and even conflicting (e.g. the diarrhoea and flushing recognised in one-third of patients with MTC have been attributed to calcitonin (CT) (Cox *et al.* 1979), serotonin (5-HT) (Leshin 1985) or prostaglandins (Williams *et al.* 1968)). However, these observations have been questioned by the finding that most patients with MTC and diarrhoea have normal serum prostaglandin levels and normal urinary 5-hydroxyindoleacetic acid excretion (Issacs *et al.* 1974). Unfortunately, because of its rarity, studies have been restricted to only a few biochemical markers; therefore, the role of other regulatory peptides could not have been addressed or correlated simultaneously.

CT (and calcitonin gene-related peptide (CGRP)) are the prime markers for MTC; however, they have been reported to be produced by many other non-thyroid cancers. Small cell bronchogenic carcinoma (Silva *et al.* 1974), phaeochromocytoma, breast and pancreatic tumours (Martin *et al.* 1980), prostatic carcinoma (Fetissof *et al.* 1986) and leukaemia (Foa *et al.* 1982) have been reported to produce CT. Similarly, CGRP has been reported in various tumours and cell lines, e.g. human lung carcinoma (Edbrooke *et al.* 1985), Ewing's sarcoma (Hoppener *et al.* 1987), phaeochromocytoma (Conlon *et al.* 1989), carcinoid (Ghatei *et al.* 1987) and prostatic adenocarcinoma (Shulkes *et al.* 1991). Therefore, the finding of a high circulating level of CT (or CGRP) is not in itself proof of MTC. With the recent increased interest in fine-needle biopsy, reliable immunocytochemical markers are in demand for firm diagnosis of MTC.

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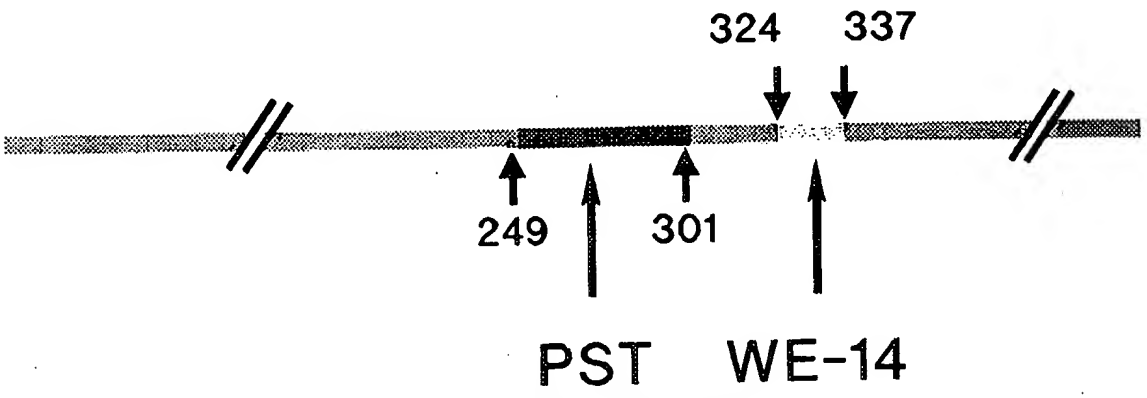

Figure 1 Schematic presentation of the CGA molecule and its fragments PST and WE-14.

This work involved the study of MTC neuroendocrine content employing RIA of the tumour extracts, supplemented by immunocytochemistry (ICC) when feasible.

The first aim was to evaluate the presence of the general neuroendocrine markers chromogranin A (CGA), neurone-specific enolase (NSE) and protein gene product 9·5 (PGP 9·5), in addition to carcino-embryonic antigen (CEA), correlating them with the prime markers of MTC, CT and CGRP. In addition, two fragments of CGA, pancreastatin (PST) and WE-14 (Fig. 1), were studied to investigate if MTC cells could process the parent peptide. Simultaneous assessment of these peptides may give an insight into the biology of MTC and validate their use as immunocytochemical markers.

A second aim of this study was to assess the relation of the tumour neuropeptide content with the pathogenesis of diarrhoea. A wide variety of possible candidates were screened including CT, CGRP and 5-HT, in addition to a battery of neuropeptides known to be secreted by APUDomas and capable of producing diarrhoea and/or vasomotor disturbances (e.g. vasoactive intestinal polypeptide (VIP), glucagon, substance P (SP), neurokinin A (NKA), gastrin and gastrin-releasing peptide (GRP)).

Subjects, Materials and Methods

Subjects

The study involved a total of 23 patients (14 males and 9 females) with histologically confirmed MTC, seen during the period 1973–1993. Six of the patients were MEN 2A and the remaining 17 were sporadic. The patients either presented to our hospital or were referred for specialist investigation and treatment. Whenever possible, tissue was taken to allow extraction for RIA for the peptides under study as well as ICC in both wax and frozen sections. However, for several patients, only pathological department archival material was available for study. Consent for the use of tissues for this research was obtained from each patient whenever possible.

Materials and methods

Tissue extraction After surgical removal, the tumour tissue was immediately snap-frozen in liquid nitrogen and stored at − 20 °C until processed. Tissue samples were extracted for peptide assay using two methods. For gastrin estimation the tissues were weighed, chopped finely and extracted using boiling phosphate buffer. The tissues were extracted twice and the volumes used in each extraction were approximately eight times the weight of tissue.

For all other peptides, extraction used ethanol/0·7 M HCl (3:1, v/v). The tissues were weighed and chopped finely in acidified ethanol, shaken for 2 h and allowed to stand at 2 °C for 24 h. The supernatant collected after centrifugation (1500 *g* for 20 min at 4 °C) was neutralised with ammonia solution and evaporated to dryness in a current of air. The dried extracts were reconstituted for assay in phosphate buffer (0·2 M, pH 7·4) (Kenny 1955).

RIA A standard RIA was used as previously described (Ardill 1979). Table 1 summarises the assay conditions and sensitivities. For tumour extracts, positive tissue controls included VIPoma, glucagonoma and gastrinoma tumours with at least 10 ng of the peptide in question/g tissue.

ICC This was performed on archival material (n=19) for CT, CGA (as well as its two fragments, PST and WE-14), NSE, PGP 9·5 and 5-HT as well as on 4% paraformaldehyde-fixed cryostat sections, where such material was available (n=3) for CGRP, SP, NKA and VIP as previously described (Johnston *et al.* 1986). Details of primary antisera employed are given in Table 2. Controls included omission of the primary antibody and preabsorption of the primary antibody with the appropriate antigen. Carcinoid tumour sections were used as positive controls for 5-HT, SP and NKA.

Table 1 Conditions and sensitivities for RIA

	Antibody dilution (final)	Sensitivity (ng/l)	Intra-assay C.V.	Inter-assay C.V.	Reference
Human CT	1:445 000	5	8·2	10·6	Beringer *et al.* 1986
Human CGRP	1:280 000	10	6·8	9·4	Shaw *et al.* 1989
GRP	1:135 000	4·5		—	McKillop 1986
PST	1:450 000	12·5	3·4	8·8	McGrath-Linden 1990
Gastrin	1:24 000	2	4·6	7·4	Ardill 1979
Glucagon	1:90 000	5	5·0	11·5	Flanagan *et al.* 1974
VIP	1:32 000	5	5·3	7·9	Ardill 1979

Table 2 Details of antisera used in ICC

	Working dilution	Source	Reference
Antibody against:			
Human CT	1:100	A	Curry *et al.* 1989
CGRP	1:300	B	Curry *et al.* 1989
CGA	1:100	C	—
PST	1:500	A	McGrath-Linden *et al.* 1991
WE-14	1:800	A	Curry *et al.* 1991
NSE	1:100	D	—
PGP 9·5	1:400	E	—
5-HT	1:500	A	Fairweather *et al.* 1990
SP	1:200	B	Curry *et al.* 1989
NKA	1:200	A	Conlon *et al.* 1986
VIP	1:100	A	Curry *et al.* 1989
CEA	1:200	F	—

A, Department of Medicine, Queen's University, Belfast, UK; B, Amersham International plc, Aylesbury, Bucks, UK; C, Incstar Hospital and Laboratory Supplies, Belfast, UK; D, Dakopatts Laboratory Supplies, Belfast, UK; E, UltraClone Ltd, Wellow, Isle of Wight, UK; F, Peninsula Laboratories, St Helens, Merseyside, UK.

Results

MTC neuroendocrine markers (Tables 3 and 4)

As expected, all the tumours expressed CT (n=23) and CGRP (n=19). CGA was expressed in 18 of the 19 tested tumours (94·7%). The fragments PST and WE-14 were expressed in 72·7% and 53·3% respectively. NSE was expressed in 94·4% and PGP 9·5 in 70·6% respectively. CEA could be tested on nine samples only, but was detected in eight of them (88·9%).

Relation of the neuropeptide content to diarrhoea

Eleven out of twenty-three patients included in this study developed diarrhoea, including nine of the twelve patients (75%) with metastatic disease and only two of the seven patients (29%) with disease limited to the thyroid. The relationship of diarrhoea to metastatic disease has been previously observed (Williams 1966). All the tumours expressed CT and CGRP. However, none expressed 5-HT (n=20), the tachykinins; SP and NKA (n=18), VIP (n=16), gastrin (n=14) or glucagon (n=11). (Tissue extracts for VIP, gastrin and glucagon were undetectable, whilst the rest of the neuropeptides were assessed by ICC.) All those tumours failing to express gastrin were shown to express GRP.

GRP was expressed in all tumours tested (n=14). The patients with GRP-positive tumours included those with (n=6) and without (n=8) diarrhoea.

Discussion

Linking MTC to the APUD system (Pearse 1968) has stimulated extensive research over the last 25 years, yet the actual relationship of the APUD system to the biology of MTC remains enigmatic. In addition to CT gene products, MTC cells have been reported to express several biochemical markers that reflect the APUD features, including somatostatin (Roos *et al.* 1981), GRP (Kameya *et al.* 1983), the enzyme L-dopa decarboxylase (Baylin & Mendelsohn 1982), CGA (Deftos *et al.* 1988) and NSE

Table 3 Clinical data and results of MTC neuropeptide expression employing RIA and/or ICC (+, present; −, absent; 0, unfeasible). The clinical data include the presence (+) or absence (−) of diarrhoea, the type of the MTC (MEN (M) or sporadic (S)), the stage at presentation (asymptomatic, detected by screening (A), localised to the thyroid, i.e. goitre (G) or with evidence of metastatic disease (M)) and status (alive without disease (1), alive with disease (2) or deceased (3))

Patient no.	Diarrhoea	Type	Stage	Status	CT	CGRP	CGA	PST	WE-14	NSE	PGP 9·5	CEA	5-HT	SP	NKA	VIP	GST	GRP	GL
1	+	M	G	3	+	+	+	+	0	+	+	0	−	−	−	−	−	+	−
2	−	M	A	1	+	+	+	−	−	+	+	+	−	−	−	−	−	+	−
3	−	M	A	1	+	+	+	+	+	+	−	0	−	−	−	−	−	+	−
4	−	M	A	1	+	+	0	+	0	0	0	0	−	−	−	−	−	+	−
5	−	M	A	1	+	+	0	+	0	0	0	0	0	−	−	−	−	+	−
6	−	M	G	1	+	+	0	+	0	0	0	0	0	−	−	−	−	+	−
7	+	S	G	2	+	+	0	+	0	0	0	0	0	−	−	−	−	+	−
8	+	S	M	3	+	+	+	+	+	+	+	0	−	−	−	−	−	+	−
9	+	S	M	3	+	+	+	+	−	+	−	0	−	−	−	−	−	+	0
10	+	S	M	3	+	+	+	+	+	+	+	0	−	−	−	−	0	0	0
11	+	S	M	2	+	+	+	0	0	+	+	0	−	−	−	−	−	+	0
12	+	S	M	2	+	0	+	+	−	+	+	+	−	0	0	0	0	0	0
13	+	S	M	3	+	+	−	−	−	−	−	+	−	−	−	−	−	+	0
14	+	S	M	3	+	0	+	−	0	+	+	+	−	0	0	0	0	0	0
15	+	S	M	3	+	0	+	+	+	+	−	0	−	0	0	0	0	0	0
16	+	S	M	3	+	0	+	+	−	+	+	0	−	0	0	0	0	0	0
17	−	S	M	3	+	+	+	−	−	+	0	−	−	−	−	0	0	0	0
18	−	S	M	3	+	+	+	+	−	+	−	0	−	−	−	−	−	+	−
19	−	S	G	1	+	+	+	+	+	+	+	+	−	−	−	0	0	0	−
20	−	S	G	1	+	+	+	+	+	+	+	+	−	0	0	0	0	0	0
21	−	S	G	2	+	+	+	−	+	+	+	0	−	−	−	−	−	+	0
22	−	S	M	3	+	+	+	−	0	0	0	+	−	−	−	−	−	+	−
23	−	S	G	2	+	+	+	+	+	+	+	+	−		−	−	0	0	0

Neuropeptides are as defined in the text and GST=gastrin and GL=glucagon.

Table 4 Summary of the results of MTC neuropeptide expression

	Total number	No. of positives	% of total
CT	23	23	100
CGRP	19	19	100
CGA	19	18	94·7
PST	22	16	72·7
WE-14	15	8	53·3
NSE	18	17	94·4
PGP 9·5	17	12	70·6
CEA	9	8	88·9

(Krisch *et al.* 1985). These were recently reported to be of no clinical significance (de Bustros & Baylin 1991).

Biological markers

The first aim of this study was to investigate some of the putative immunocytochemical markers and decide if their existence could be of any diagnostic significance. Because MTC is usually diagnosed in retrospect from pathological specimens or using fine-needle aspiration biopsy, the availability of reliable immunocytochemical markers may provide the opportunity to confirm the diagnosis pre-operatively. As expected, all the tested tumours expressed CT and CGRP which have been established as standard markers for MTC (de Bustros & Baylin 1991).

Our results confirm the previous observation of CGA being expressed by MTCs (Deftos *et al.* 1988). This work was further extended to examine whether MTCs actually process CGA which is now believed to be the precursor of other peptides including PST and WE-14 (Fig. 1). PST was detected in almost three-quarters and WE-14 in half of the examined tumours. This would suggest that CGA may be an important marker for MTC.

NSE is an enzyme specific for neuronal cells whose presence in APUD cells supports the postulate of a common neural origin of all these cells (Schmechel *et al.* 1978). The detection of NSE in most of our patients, together with the reported rise in serum NSE in parallel with a large tumour mass and the serum CT level (Pacini *et al.* 1986) would suggest that it can also be used as a marker for MTC.

PGP 9·5 is another general neuroendocrine marker with extensive co-localisation with NSE, but with a totally different amino acid sequence (Thompson & Day 1988).

The coexistence of these three neuroendocrine markers in the majority of cases, although not specific to MTC, strongly implies the full expression of APUD characteristics in MTC and consequently means that their use as markers might be of clinical significance.

Patient no. 13, who had the shortest postoperative survival (14 months), was the only one in our group who failed to express CGA (or its fragments), NSE or PGP 9·5. Although by no means conclusive, this isolated finding might suggest that the loss of expression of these neuroendocrine markers is accompanied by a more aggressive course. Further work is required in this area.

The expression of CEA in almost all of MTCs would imply the relevance of this peptide, not only as a biochemical but also as an immunocytochemical marker.

Neuropeptide content in relation to diarrhoea

The second aim of this work was to investigate the possible role of various products of the APUD cells on the diarrhoea of MTC.

5-HT, a vasoactive compound with a neurocrine effect on the gut, has been firmly implicated in the pathogenesis of the diarrhoea of the carcinoid syndrome. As an APUDoma, MTC is well known for its ability to produce 5-HT (de Bustros & Baylin 1991). However, the role of 5-HT in the aetiology of diarrhoea, found in some 30% of cases, is controversial; some investigators have reported 5-HT, using ICC, in 15 of 25 medullary cancers, although no clinical correlations were made (Holm *et al.* 1985). Others have reported normal circulating levels of 5-HT in MTC patients with diarrhoea. Nevertheless, it is still reputed to contribute to diarrhoea. All the tested tumours in our group proved negative. Therefore, regardless of previous reports of 5-HT in MTCs, our results reasonably exclude 5-HT as a mediator of the diarrhoea.

SP, NKA, VIP and glucagon are well-recognised products of the APUD cells and are implicated in diarrhoea syndromes seen with VIPoma and carcinoid tumours. Our tissue extraction screening, supplemented by ICC, showed an absence of all of these peptides. They would appear to have no role in the diarrhoea of MTC.

The secretion of gastrin (another APUD cell product), implicated in the diarrhoea of the Zollinger–Ellison syndrome, is stimulated by GRP (Mulvihill & Debas 1994). GRP was found to be present in MTC by RIA and by ICC. We could not detect gastrin in any of 14 tumours extracted, but GRP was found in all those cases. A working hypothesis considered that MTC released GRP which stimulated the production of gastrin. This could explain the diarrhoea as GRP was demonstrated in the extracts of all those with diarrhoea. The patient (patient no. 9) with the highest level was reported to have Zollinger–Ellison syndrome and in another three patients pancreatic enlargement was documented. Many points, however, require to be elucidated. For example, is the serum level of gastrin elevated in parallel with GRP and will it normalise after MTC resection? Also, GRP was recovered from a metastatic MTC in which diarrhoea was not a feature.

In conclusion, a number of neuroendocrine markers were shown to be expressed in MTC tumours from a variety of stages including both sporadic and MEN 2A-associated. No APUD cell products previously thought to

mediate MTC-associated diarrhoea were identified. Expression of GRP in some patients may mediate diarrhoea by stimulating gastrin.

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